METHOD OF CHROMATOGRAPHIC ANALYSIS OF A PROTEIN SOLUTION

Field of the invention

This invention relates to methods for the analysis of proteins.

More specifically, it relates to the analysis of proteins by chromatographic methods (e.g. HPLC). By "analysis" of proteins is meant here both the quantitative determination and the assessment of purity of a protein.

Background of the invention

Proteins in pharmaceutical products must be analysed to quantify the protein and to ensure purity. This permits correct and reproducible dosing to the patient.

Analytical techniques are necessary to quantify the pharmaceutical protein as well as impurities, aggregates, and degradation products. In the case of multimeric proteins, such as dimers, analytical techniques are required to detect the presence and extent (i.e. quantity) of dissociation.
Such analytical techniques should be precise (i.e. the degree of variation when the same sample is tested multiple times should be low), and accurate (i.e. the measured value should be as close as possible to the actual value). Reproducibility is also important.

An example of an analytical assay that may be used with proteins is size exclusion chromatography (SEC), in which a protein in aqueous solution is

passed over a solid or gel phase that separates mixtures of protein by differences in their molecular weight. The resulting chromatogram shows one or more peaks associated with the protein(s) in a sample, which may be identified by molecular weight. The area under a peak associated with a given protein can be used to quantify the amount of that protein in the sample. The shape of the peak may be used to assess purity.

Another example of an analytical assay that may be used with proteins is high performance liquid chromatography (HPLC), in particular reverse phase high performance liquid chromatography (RP-HPLC). A sample containing the pharmaceutical protein is passed through a column which separates the protein from any impurities. The protein and any impurities elute as peaks on a chromatogram. As with SEC, the area under a peak associated with a given protein can be used to quantify the amount of that protein in the sample. The shape of the peak may be used to assess purity.

In chromatographic methods, such as those mentioned above, the protein must be dissolved in an aqueous solvent and diluted to an extent acceptable for the chromatographic system used. During handling of the aqueous protein solution, the risk exists that part of the protein is lost by adsorption to handling and containment equipment, such as glass or plastic walls of capillaries, test-tubes, beakers, syringes, etc., making quantitation of protein difficult. The adsorption of protein leads to variations in the results that detract from the assay precision, accuracy and reproducibility

Surfactants have been used in the purification of proteins by SEC, and in assays in which the molecular weight of a new protein is determined, see for example EP 0 530 937 and DE 39 17 949.

It would be desirable to have a chromatographic method of protein analysis for quantifying protein and/or assessing purity, in which assay precision, accuracy and reproducibility are improved by avoiding variations due to protein adsorption.

Summary of the invention

It has now been discovered that a surfactant of the class of Poloxamers avoids protein loss and at the same time does not interfere with the chromatographic analysis.

In a first aspect, the invention provides, in a method of chromatographic analysis of a protein sample solution, the improvement consisting in adding a Poloxamer to the protein sample solution.

In a second aspect, the invention provides, in a method of chromatographic analysis of a protein including the step of preparing a diluted sample for bringing the protein concentration to a level acceptable for the chromatographic system used, the improvement consisting in adding a Poloxamer to the diluted sample solution.

In a third aspect, the invention provides a method for the chromatographic analysis of the purity and/or quantity of a protein in a sample, the method comprising a step of preparing the sample to contain a Poloxamer.

Detailed description of the invention

The inventors have found that by including a Poloxamer in a protein solution to be assayed for purity and protein content, protein adsorption is decreased, leading to increased assay precision, accuracy and reproducibility.

In the context of the present application, the term "analysis" is meant to encompass an analytical process whereby the purity of and/or quantity (e.g. concentration) of a protein in a sample is determined, preferably the quantity. In a preferred embodiment, the method of the invention comprises a method for the analysis of the purity and/or quantity of a protein in a sample, the method comprising a step of preparing the protein sample to contain a Poloxamer, and performing a step of chromatography, preferably a step of SEC or RP-HPLC. Preferably the step of chromatography is followed by a step of data manipulation to determine purity and/or quantity of the protein. The quantity of protein may be determined using data from calibration with a standard. Calibration may be carried out before or after analysis of the sample.

Poloxamers are block copolymers made of poly(oxyethylene)-poly(oxypropylene) blocks with M.W. ranges from 1,000 to > 16,000. Their main characteristic is that the poly(oxyethylene) segments are hydrophobic

and the poly(oxypropylene) segments hydrophilic. These substances behave as non-ionic surfactants and are generally known with the commercial name "Pluronics".

Many grades of Pluronics at various Molecular Weight and concentration ranges can be used in accordance with this invention.

As mentioned above, the Poloxamer (Pluronic) surfactants are block copolymers of ethylene oxide (EO) and propylene oxide (PO). The propylene oxide block (PO) is sandwiched between two ethylene oxide (EO) blocks.

$$HO{-}(CH_2CH_2O)_x\text{-}(CH_2\overset{\displaystyle CH_3}{\overset{|}{C}}HO)_y\text{-}(CH_2CH_2O)_x\text{-}H$$

$$\boxed{\text{EO}}\boxed{\text{PO}}\boxed{\text{EO}}$$

Pluronic surfactants are synthesised in a two-step process:

1. A hydrophobe of the desired molecular weight is created by the controlled addition of propylene oxide to the two hydroxyl groups of propylene glycol; and

2. Ethylene oxide is added to sandwich the hydrophobe between hydrophilic groups.

In Pluronic® F77, the percentage of polyoxyethylene (hydrophile) is 70%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 2,306 Da.

In Pluronic F87, the percentage of polyoxyethylene (hydrophile) is 70%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 2,644 Da.

In Pluronic F88, the percentage of polyoxyethylene (hydrophile) is 80%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 2,644 Da.

In Pluronic F68, the percentage of polyoxyethylene (hydrophile) is 80%, and the molecular weight of the hydrophobe (polyoxypropylene) is approximately 1,967 Da.

Typical properties of Pluronic F77 are listed below:

Average Molecular Weight: 6600;

Melt/pour point: 48°C ;

Physical Form @ 20°C : solid;

Viscosity (Brookfield) cps: 480 [liquids at 25°C, pastes at 60°C and solids at 77°C];

Surface tension, dynes/cm @ 25°C;

0.1% Conc. : 47.0

0.01% Conc. : 49.3

0.001% Conc.: 52.8

Interfacial tension, dynes/cm @ 25°C vs. Nujol;

0.1% Conc. : 17.7

0.01% Conc. : 20.8

0.01% Conc. : 25.5

Draves Wetting, Seconds 25°C

1.0% Conc.: > 360

0.1% Conc.: > 360

Foam Height

Ross Miles, 0.1%, mm @ 50°C: 100

Ross Miles, 0.1%, mm @ 26°C: 47

Dynamic, 0.1%, mm @ 400 ml/min: > 600

Cloud point in aqueous solution, °C

1% Conc.: >100

10% Conc.: >100

HLB (hydrophile-lipophile balance): 25

Typical properties of Pluronic F87 are listed below:

Average Molecular Weight: 7700;

Melt/pour point: 49°C ;

Physical Form @ 20°C : solid;

Viscosity (Brookfield) cps: 700 [liquids at 25°C, pastes at 60°C and solids at 77°C];

Surface tension, dynes/cm @ 25°C;

0.1% Conc. : 44.0

0.01% Conc. : 47.0

0.001% Conc.: 50.2

Interfacial tension, dynes/cm @ 25°C vs Nujol;

0.1% Conc. : 17.4

0.01% Conc. : 20.3

0.01% Conc. : 23.3

Draves Wetting, Seconds 25°C

1.0% Conc.: > 360

0.1% Conc.: > 360

Foam Height

Ross Miles, 0.1%, mm @ 50°C: 80

Ross Miles, 0.1%, mm @ 26°C: 37

Dynamic, 0.1%, mm @ 400 ml/min: > 600

Cloud point in aqueous solution, °C

1% Conc.: >100

10% Conc.: >100

HLB (hydrophile-lipophile balance): 24

Typical properties of Pluronic F88 are listed below:

Average Molecular Weight: 11400;

Melt/pour point: 54°C ;

Physical Form @ 20°C : solid;

Viscosity (Brookfield) cps: 2300 [liquids at 25°C, pastes at 60°C and solids at 77°C];

Surface tension, dynes/cm @ 25°C;

0.1% Conc. : 48.5

0.01% Conc. : 52.6

0.001% Conc.: 55.7

Interfacial tension, dynes/cm @ 25°C vs Nujol;

0.1% Conc. : 20.5

0.01% Conc. : 23.3

0.01% Conc. : 27.0

Draves Wetting, Seconds 25°C

1.0% Conc.: > 360

0.1% Conc.: > 360

Foam Height

Ross Miles, 0.1%, mm @ 50°C: 80

Ross Miles, 0.1%, mm @ 26°C: 37

Dynamic, 0.1%, mm @ 400 ml/min: > 600

Cloud point in aqueous solution, °C

1% Conc.: >100

10% Conc.: >100

HLB (hydrophile-lipophile balance): 28

Typical properties of Pluronic F68 are listed below:

Average Molecular Weight: 8400;

Melt/pour point: 52°C ;

Physical Form @ 20°C : solid;

Viscosity (Brookfield) cps: 1000 [liquids at 25°C, pastes at 60°C and solids at 77°C];

Surface tension, dynes/cm @ 25°C;

0.1% Conc. : 50.3

0.01% Conc. : 51.2

0.001% Conc.: 53.6

Interfacial tension, dynes/cm @ 25°C vs Nujol;

0.1% Conc. : 19.8

0.01% Conc. : 24.0

0.01% Conc. : 26.0

Draves Wetting, Seconds 25°C

1.0% Conc.: > 360

0.1% Conc.: > 360

Foam Height

Ross Miles, 0.1%, mm @ 50°C: 35

Ross Miles, 0.1%, mm @ 26°C: 40

Dynamic, 0.1%, mm @ 400 ml/min: > 600

Cloud point in aqueous solution, °C

1% Conc.: >100

10% Conc.: >100

HLB (hydrophile-lipophile balance): 29

Other polymers having properties similar to those listed above may also be used in the methods of the invention. The preferred surfactant is Pluronic F68 (Poloxamer 188), and surfactants having similar properties.

Therefore, this invention relates to an improved method of chromatographic analysis of a protein including the step of preparation of a protein sample with a protein concentration acceptable for the chromatographic system used, the

improvement consisting in adding a Poloxamer, preferably Pluronic F68, to the protein sample solution.

The method can be used essentially with any protein, for example insulin, etanercept, Factor VIII, growth hormone (Somatotropin), antibodies (such as infliximab), leukaemia inhibitory factor (LIF, enfilermin), an interleukin, such as interleukin-6 (Atexakin alpha), tumour necrosis factor binding protein (TBP-1, Onercept), interleukin-18 binding protein (IL-18 BP, Tadekin), anti-CD11a (Efalizumab). In a preferred embodiment, the protein is a glycoprotein, such as erythropoietin (EPO), darbepoietin alfa, human protein C, interferons (such as interferon beta 1a, 1b, particularly preferably interferon-beta-1a), alpha galactosidase A. Preferably the protein is a dimeric protein, i.e. composed of subunits (including heterodimeric), particularly preferably a dimeric or heterodimeric glycoprotein, for example thyroid-stimulating hormone (TSH), and the gonadotrophins: follicle-stimulating hormone (FSH), luteinising hormone (LH), and chorionic gonadotrophin (CG). Preferably these proteins are human proteins.

In the Examples which follow, Pluronic F68 (Poloxamer 188) is used at the concentration of 100 µg /ml in ultra-pure water. It is however understood that the use of different grades of Pluronic and different concentrations of the same is encompassed by the present invention.

EXAMPLE1

Improved sample preparation in the SEC method of quantitative determination of FSH

The purpose of this study was to qualify an improved sample preparation in the Size Exclusion Chromatography (SEC) method for protein content in a preparation containing rec-FSH (Fertinex, in this case Fertinex 75 IU). The modification implemented was the use of a solution including 100 µg/ml Pluronic F68 in ultra-pure water for preparation of all protein solutions (sample, control sample and standard) in order to control losses of protein due to adsorption.

Samples consisting of mixtures of heterodimeric FSH (FSH is composed of an α-subunit and a β-subunit) and dissociated FSH were prepared and analysed. The method allowed the quantitation of heterodimeric FSH and free subunits.

This study shows that a single point calibration curve using drug product reference standard is suitable to determine protein content with a good total precision of 2.0% and that the method is linear within the range tested (26.6 to 160 µg/ml).

In addition, both Waters and Varian HPLC systems can be used as shown during the study. The difference in the mean protein content results of all batches is lower than the total precision of the method.

It is important to emphasize that this change did not have any impact on the original SEC method itself.

More specifically, the modification of the sample and standard preparations were as follows:

- Use of a 100 µg/ml Pluronic F68 in ultra-pure water for the preparation of the samples and reference standard.
- Blank solution used through the analytical sequence is the 100 µg/ml Pluronic F68 solution.
- The preparation for SST control sample as well as control samples injected over the analytical session are performed by reconstituting and pooling enough ampoules to allow the injections of the system suitability as well as for the controls which are injected throughout the analytical session.

Materials and equipment

Materials

Control sample: highly purified urinary FSH (u-FSH-HP) batch 17304010

Test samples: 75 IU u-FSH-HP batch nos. 17301010, 17315040 and 17318040.

Equipment

HPLC pump mod. 600E or 626 [Waters]

UV detector mod. 486 or 996 [Waters]

Column: Progel TSK G2000 SW 60 cm X 7.5 mm [Supelco]

Procedure

The column was conditioned for an hour with the mobile phase (phosphate buffer 0.1 M; Na_2SO_4 0.1 M, pH 6.70), at a flow rate of 0.70 ml/minute. The column was maintained at approximately 4°C throughout the analysis.

For analysis, the following solutions were used:

Standard solution: a solution of highly purified FSH (0.041 μg/μl) was prepared with Pluronic F68 (100 μg/ml).

Sample solutions: the sample solutions were prepared to have varying amounts of heterodimeric FSH (Fertinex) and dissociated FSH, in a solution containing Pluronic F68 (100 μg/ml).

Control solutions: control solution was prepared using FSH dissolved in a solution containing Pluronic F68 (100 μg/ml).

The standard and sample solutions were injected (100 μl), and the column was eluted at a constant flow rate of 0.70 ml/min. Detection was by UV absorption at 214 nm. Area under the peaks was used to determine heterodimeric FSH and the respective FSH subunits.

Results

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels : repeatability (or intra-assay precision), intermediate precision and reproducibility. During this study, repeatability, intermediate precision as well as reproducibility of the assay were addressed.

In five independent analytical sessions, three Fertinex drug products batches were quantified against the standard (calibration curve).

With the results of Fertinex 75 IU presented in Table 1, an analysis of variance (ANOVA) Nested design was used to estimate the repeatability (or intra-assay precision), intermediate precision and reproducibility (total precision) of the analytical method. The total number of results under study was 45. The results obtained were as follows:

- Repeatability (or intra-assay precision): 1.3%
- Intermediate precision: 1.0 %
- Reproducibility (total precision): 1.6%

Overall results are tabulated in the following Table 1:

Table 1: Protein content results (µg/ampoule) obtained with calibration curve standard

Batch number	Run 1	Run 2	Run 3	Run 4	Run 5
17301010	6,41	6,42	6,32	6,56	6,41
17315040	6,13	6,06	5,94	6,11	6,15
17318040	5,54	5,58	5,49	5,59	5,51
Control sample	6,00	6,18	6,04	6,21	6,07

The standard results obtained during the precision and ruggedness studied were used to address the linearity and range of the analytical method. The approach for the statistical analysis was to check variance homogeneity (Cochran C and Bartlett tests), lack of fit and perform regression analysis (correlation coefficient). Run 1 to Run 5 were performed with a Waters systems whereas Run 6 to Run 8 were performed with a Varian system.

Table 2: Statistical results for linearity

	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8
Cochran's C test	0,99	0,98	0,99	0,99	0,99	0,98	0,96	0,98
Bartlett's test	0,99	0,98	0,99	0,99	0,99	0,98	0,96	0,98
Lack of fit	0,97	0,18	0,83	0,92	0,81	0,89	0,03	0,07
Slope	1,0058	0,9926	1,0028	1,0066	0,9940	0,9903	1,0202	0,9888
Intercept	6,1557	6,2238	6,1574	6,2099	6,2249	5,5055	5,7437	5,5214
Correlation coeff.	1,0000	0,9999	0,9999	0,9999	1,0000	0,9999	0,9998	0,9999

As can be seen, the p-values of Cochran C and Bartlett tests are always greater than 0.05 which means that there is no statistically significant difference amongst the standard deviations at 95% confidence level and a correlation coefficient higher than 0.9980 is always met.

Lack of fit to determine whether linear regression is an adequate model to describe the observed data was performed. Based on this statistical test, linear regression resulted to be the best model to describe the data even if the p-value of Run 7 is not greater than 0.05. For that particular analytical session, the linear regression is still the best model with a 99.96% fit. Other models, such as square root and exponential models were tested and did not show a better fit of the data observed.

EXAMPLE 2

Improved sample preparation and spiking procedure in the SEC method of purity determination of FSH

In a spiking experiment, known amounts of either heterodimeric FSH or dissociated FSH were added to a sample of FSH ("spiking"). The resulting peaks for heterodimeric and/or dissociated subunits were evaluated for % recovery and for % purity.

The modification implemented in the analytical method is the use of a solution including 100 µg/ml Pluronic F68 in ultra-pure water for preparation of all protein solutions (sample, spike solution) to control losses of protein due to adsorption. The modifications were made specifically to increase the precision of the method, without impacting the chromatography of the method, to enable a more precise and accurate purity determination.

In the frame of this study, determination of the precision of the method was also investigated. The precision of the method (Total precision 1.8%) was slightly improved when compared to the precision of the method observed during validation of the analytical method without introduction of Pluronic F68 (Precision 2%). Routine recovery of the spike solutions at 100% indicates a good accuracy.

An additional spiking experiment was performed to further determine accuracy of the method. It was observed that different area under the peak is obtained for the spiking solution with and without Pluronic F68. The area of the spiking solution with Pluronic F68 in the sample preparation is approximately two times greater than the spiking solution prepared without the use of Pluronic F68.

It is believed that the different areas observed are due to adsorption of the dissociated sub-units on the polypropylene material used for sample preparation.

It can be seen in the following Tables 3 and 4 that the recovery of spiked free sub-units of FSH is within the range 95% - 105%. In addition it can also be seen that the precision [reported as coefficient of variability (CV%)] over the five runs are ranging from 1.0% to 1.5% for both purity and recovery of spike. The lower the CV%, the lesser the variability between runs.

This is a substantial improvement over samples prepared without Pluronic F68.

Table 3: %Recovery of spike results

Batch#	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	CV %
17343109	98,0%	98,1%	100,3%	99,6%	99,4%	99,1%	1,0%
17349129	98,9%	99,6%	102,1%	100,7%	102,2%	100,7%	1,5%
17301010	99,8%	100,7%	102,0%	99,2%	99,8%	100,3%	1,1%
17304010	99,8%	102,4%	101,6%	101,0%	99,9%	100,9%	1,1%

Table 4: %Purity of spike results

Batch#	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	CV %
17343109	97,0%	94,4%	93,9%	96,3%	96,3%	95,6%	1,4%
17349129	96,2%	93,4%	93,3%	95,5%	94,4%	94,6%	1,4%
17301010	96,8%	95,0%	94,1%	96,9%	97,2%	96,0%	1,4%
17304010	95,9%	93,6%	94,0%	95,9%	97,0%	95,3%	1,5%

In the frame of another study protocol, the analysis of drug product batches with and without the use of Pluronic F68 was performed. One of the results is that the area under the peak of the spiking solution (dissociated r-hFSH) is approximately multiplied by two with the introduction of Pluronic F68. This phenomenon is most probably due to adsorption of free sub-units on the material used during sample preparation if Pluronic F68 is not present. To determine if this increase of area has an impact on the analysis, a spiking experiment at three levels without and with Pluronic F68 was performed. The following samples were tested in duplicate:

1.Sample preparation with Pluronic F68

- Sample without spiking solution;
- Sample spiked with dissociated r-hFSH (2 µg by injection);
- Spiking solution (2 µg dissociated r-hFSH by injection);
- Sample spiked with dissociated r-hFSH (1.5 µg by injection);
- Spiking solution (1.5 µg dissociated r-hFSH by injection);
- Sample spiked with dissociated r-hFSH (1 µg by injection);
- Spiking solution (1µg dissociated r-FSH by injection).

The results are presented in table 5:

Table 5: Results of sample preparations using Pluronic F68: area under peak

Spiking level	Area of spiking solution	% Recovery of areas	% Purity	% Recovery of spike
100% (2 µg/inj)	2037915	N/A	95%	101%
75% (1.5 µg/inj)	1545486	76%	94%	101%
50% (1 µg/inj)	1001055	49%	94%	102%

From the above table, it can be seen that the addition of Pluronic F68 gives good results in term of % recovery of spike, being close to the

theoretical recovery (100%) and % Recovery of areas. This latter parameter is calculated by dividing the area of the spiking solution at the defined spiking level by the area of the spiking solution at 100%. The result (i.e. 76% for spiking with 1.5 µg/injection) is compared with the theoretical spiking level performed (i.e. 75%).

2. Sample preparation without Pluronic F68

The following samples were tested in duplicate:

- Sample without spiking solution;
- Sample spiked with dissociated r-hFSH (2 µg by injection);
- Spiking solution (2 µg dissociated r-hFSH by injection);
- Sample spiked with dissociated r-hFSH (3 µg by injection) ;
- Spiking solution (3 µg dissociated r-hFSH by injection);
- Sample spiked with dissociated r-hFSH (4 µg by injection);
- Spiking solution (4 µg dissociated r-hFSH by injection)

Results are shown in the following Table 6:

Table 6 : Results of sample preparations without Pluronic F68: area under peak

Spiking level	Area of spiking solution	% Recovery of areas	% Purity	% Recovery
100% (2 µg/inj)	922106	N/A	90%	105%
150% (3 µg/inj)	1744753	189%	89%	104%
200% (4 µg/inj)	2702520	292%	89%	104%

For the results obtained without the use of Pluronic F68 in the sample preparation, the recovery of the spike solution deviates 4-5% from the theoretical recovery (100%). For % recovery of areas, the results deviate significantly from the theoretical spiking level performed, due to a too low area of the spike solution.

It is believed that the different results obtained with and without Pluronic F68 can be explained by the adsorption of the test samples on the material used during sample preparation if Pluronic is not included in the assay solutions. The area under the peak of spiking solution obtained for the same spiking level (100%) is approximately two times greater when Pluronic F68 is present and avoids the adsorption.

The adsorption of free sub-units can be calculated by the difference between the area under the peak of the spiking solution with Pluronic F68 and the area of the spiking solution without Pluronic F68 at 2 µg per injection (100%) spiking level [i.e. subtracting the area under the peak at a spiking level of 100% without Puronic F68 (Table 6: 922106) from the area under the peak at a spiking level of 100% with Pluronic F68 (Table 5: 2037915)]. The difference corresponds to an area of 1'115'809. This area reflects the amount of dissociated sub-units adsorbed during sample preparation when Pluronic F68 is not present, and can be used to correct the area obtained in absence of Pluronic F68. The recoveries of area calculated taking into account this correction can be seen in table 7. The data are closer to the theoretical spiking level.

Table 7: %Recovery of area with and without taking into account adsorption (in the absence of Pluronic F68)

Spiking level	%Recovery of areas taking into account adsorption	
	No	Yes
3 µg/inj (150%)	189%	140%
4 µg/inj (200%)	292%	187%

The purity results presented in tables 5 and 6 differ without and with the introduction of Pluronic. This difference is also believed to be an effect of adsorption phėnomenon. Percent purity is significantly more accurate when Pluronic F68 is used.

Several product batches were tested by SEC for purity without and with introduction of Pluronic F68 in sample preparation. The results can be seen in table 8:

Table 8: %Recovery and %Purity results of drug product tested without and with Pluronic F68

Batch#	Without Pluronic		With Pluronic	
	%Recovery	%Purity	%Recovery	%Purity
17353066	98%	89%	96%	94%
17303019	101%	91%	100%	97%
17306019	104%	91%	97%	98%
17309029	100%	90%	100%	97%
17323049	101%	92%	98%	97%
17324049	98%	94%	97%	99%
17340089	100%	93%	99%	97%
17341089	101%	91%	98%	98%
17345119	100%	90%	100%	96%
17305019	105%	88%	99%	101%
17315039	99%	91%	101%	95%
17302019	100%	87%	99%	96%
17501019	106%	86%	99%	96%
Mean	101,0%	90,2%	98,7%	97,0%
Standard deviation	2,5%	2,3%	1,4%	1,8%
CV %	2,5%	2,5%	1,5%	1,8%

Purity and recovery of spiking solution

Based on the data presented in Table 8 above, it can be seen that the difference between the recovery without and with introduction of Pluronic is about 2%. This difference is statistically significant when an ANOVA at 95% confidence level is performed (p-value 0.008). In addition, when looking at the same table one can also see a statistically significant difference of approximately 7% in purity with and without introduction of Pluronic F68 (ANOVA p-value 1.2 10^{-8}).

Furthermore, it can be seen from Table 8 that the CV% is lower when Pluronic F68 was included in the sample solution [2.5% without Pluronic F68 VS 1.5 and 1.8% with Pluronic F68]. A lower CV% indicates a lesser degree of variability between runs.

These statistical differences in purity and recovery of spiking solution can be explained by adsorption phenomenon which occurs during sample preparation when Pluronic F68 is not used (see the following Table 9).

In Table 9 and subsequent Tables, "Dimers and Aggregates" refers to aggregated FSH molecules and dimers of heterodimeric FSH that are generally considered to be undesirable.

Table 9 : Area variability: area under peaks of heterodimeric FSH, free subunits and dimers and aggregates

Batch #	Total area of FSH spiking solution				Total area of unspiked sample				Dimers and aggregates area of sample			
	Area		CV%		Area		CV%		Area		CV%	
Pluronic	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
17353066	1409832	2658545	2,2	1,7	7377193	7767243	0,4	2,5	85841	90638	10,33	3,92
17303019	1438014	2513326	0,5	0,2	6136762	6312492	4,8	2,2	65913	64896	2,28	3,05
17306019	1438014	2513326	0,5	0,2	6029360	6474462	1,2	1,2	69387	65633	3,06	6,49
17309029	1121632	2380100	3,1	2,4	6554845	6906608	0,5	0,5	74694	83950	4,36	5,06
17323049	1300382	2429203	1,2	1,5	5692277	6157225	1,8	1,5	64801	67053	5,47	7,62
17324049	1300382	2429203	1,2	1,5	5856669	6129612	4,6	1,5	61088	58535	5,49	0,72
17340089	1449322	2564021	2,3	3,4	6316392	6577138	1,7	0,7	55831	64916	1,93	3,53
17341089	1449322	2564021	2,3	3,4	6311904	6658876	1,3	0,4	51410	66879	6,79	2,36

Table 9 : Area variability: area under peaks of heterodimeric FSH, free subunits and dimers and aggregates

Batch #	Total area of FSH spiking solution				Total area of unspiked sample				Dimers and aggregates area of sample			
	Area		CV%		Area		CV%		Area		CV%	
Pluronic	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
17345119	1121632	2380100	3,1	2,4	5678718	6094436	0,5	0,3	61319	71681	5,86	0,98
17305019	1416915	2609854	3,5	0,8	6105716	6366397	3,4	1,3	81412	76271	8,77	1,96
17315039	1207140	2528909	7,4	5,7	6629111	7129130	1,4	0,6	69514	65511	1,74	3,77
17302019	1207140	2528909	7,4	5,7	6306795	6975041	3,8	0,6	97979	94241	5,28	8,59
17501019	1416915	2609854	3,5	0,8	7416153	7580193	1,1	2,1	69441	67857	6,4	6,1
Mean	1328972	2516105	2,9	2,3	6339377	6702219	2,0	1,2	69895	72159	5,2	4,2
CV %	9,5%	3,5%	76%	81%	8,7%	8,1%	77%	63%	18%	15%	50%	59%

Table 9 : Area variability (continued)

Table 9 : Area variability (continued)								
Batch #	Dissociated FSH area of spiked sample				Total area of spiked sample			
	Area		CV%		Area		CV%	
Pluronic	No	Yes	No	Yes	No	Yes	No	Yes
17353066	2132665	3052448	2,5	1,08	8618890	9989197	0,92	0,41
17303019	1908454	2640090	4,5	0,43	7634720	8866597	1,52	0,83
17306019	1920007	2564678	1,2	0,81	7739564	8723677	1,12	0,58
17309029	1697129	2520633	6,0	2,01	7695502	9270545	2,16	1,48
17323049	1697821	2542802	1,8	1,14	7033218	8410312	1,14	1,09
17324049	1596022	2439971	6,4	2,61	7022864	8324957	2,32	0,81
17340089	1862023	2678583	3,2	1,27	7763745	9078480	0,79	1,49

Table 9 : Area variability (continued)

Batch #	Dissociated FSH area of spiked sample				Total area of spiked sample			
	Area		CV%		Area		CV%	
Pluronic	No	Yes	No	Yes	No	Yes	No	Yes
17341089	1977742	2642383	1,3	1,16	7846421	9034549	0,31	0,27
17345119	1616907	2555312	5,7	1,80	6808816	8480285	1,21	1,17
17305019	2044400	2479940	1,2	2,99	7863722	8851837	3,27	0,95
17315039	1722445	2785597	5,0	0,08	7721964	9750217	2,01	0,41
17302019	1943343	2681371	11,0	2,72	7543478	9363087	2,68	2,07
17501019	2354166	2847934	0,4	0,5	9364548	10138501	0,2	0,35
Mean	1882548	2648596	3,9	1,4	7742881	9098634	1,5	0,9
CV %	12%	6,3%	77%	65%	8,6%	6,4%	61%	59%

It can be seen from Table 9 that the precision (CV%) of the areas used for the purity and recovery of spiking solutions calculation is almost always better with Pluronic F68 than without the introduction of Pluronic F68 [lower CV% indicates improved precision].

The adsorption rate of dimers and aggregates, free sub-units and monomer are different as one can see when calculating the ratio mean area with Pluronic F68/ mean area without Pluronic F68. As can be seen in table 10, the %Area increase with Pluronic F68 is not constant depending on the area considered.

Table10: %Area increase with introduction of Pluronic F68

Area considered	%Area increase
Total area of spiking solution	6%
Total area of unspiked sample	89%
Dimers and aggregates areas of unspiked sample	41%
Dissociated sub-units area of spiked sample	3%
Total area of spiked sample	18%

To determine purity, spiking of free sub-units must be performed due to the fact that the free sub-units peak is not resolved from main peak during testing of sample preparation. The %Purity (or %Monomer) is expressed by the formula below:

$$\%\text{Purity} = \frac{A+B-C-D}{A}$$

Where: A : Total area of sample without spike

B : Total area of spiking solution

C : Sub-units peak area of sample spiked

D : Dimers and aggregates in sample without spike

The acceptance criteria for recovery of spike is expressed as follows:

$$\%\text{Recovery} = \frac{E}{A+B}$$

Where: A : Total area of sample without spike

B : Total area of spiking solution

E : Total area of spiked sample

Taking the above formulas into consideration, calculation of purity with and without introduction of Pluronic F68 can be performed. In addition, to take into consideration the effect of Pluronic F68, calculations can

also be done with areas without introduction of Pluronic F68 taking into account the adsorption effect. The formulas will be the following :

$$\%\text{Purity corrected} = \frac{A \times Ra + B \times Rb - C \times Rc - D \times Rd}{A \times Ra}$$

$$\%\text{Recovery corrected} = \frac{E \times Re}{A \times Ra + B \times Rb}$$

Where: A : Total area of sample without spike

B : Total area of spiking solution

C : Sub-units peak area of sample spiked

D : Dimers and aggregates in sample without spike

E : Total area of spiked sample

Rx: Ratio mean area X with Pluronic F68 divided by mean area X without Pluronic F68.

EXAMPLE 3

Interferon beta-1a assay by RP-HPLC: Qualification of a One Standard point approach versus a standard curve approach

This Example shows how, in the case of Interferon beta-1a assay by RP-HPLC, it was possible to modify a standard curve approach, hereinafter referred to as "current assay", to a One Standard Point approach by applying the improved method of this invention, that is, by using a Pluronic surfactant to avoid sample losses.

In the drug substance sample preparation for the improved method of the invention, Interferon beta-1a was diluted 1 to 7 using as the dilution buffer 0.05M sodium acetate containing 0.1% Poloxamer 188 (Pluronic F68) at pH 3.8 instead of 0.05M sodium acetate at pH 3.8 without Poloxamer.

The optimized RP-HPLC assay was qualified according to the ICH Guidelines and the following characteristics were addressed:

• Range of linearity: The linearity was verified for the injection of Interferon-beta-1a in the range of 3.3 to 8.8 µg, with an intercept statistically not different from zero. These results support the One Standard Point approach.

• Precision: The precision of the optimized assay using the Drug Product Standard and the One Standard Point approach was compared to the precision obtained with the current assay using the Drug Substance Standard and the Standard curve approach. The overall precision obtained for one replicate with the optimized assay using Pluronic F68 (pooled CV of 1.2 % for all Drug Substances and Drug Products samples) was shown to be better than the precision of 1.9 % obtained for one replicate with the current assay, not using Pluronic F68.

• Specificity: the components that can be observed in the optimized assay are already present in the Drug Substance and Drug Product formulations analyzed in the current assay which was validated for specificity. The presence of Poloxamer 188 in the new Standard was demonstrated to not

interfere in the optimized assay. As no additional components are introduced in the optimized assay, the optimized assay is therefore specific.

• Accuracy: The accuracy of the optimized assay was assessed by comparison of the results obtained with the optimized assay versus the results obtained with current assay, these last results being considered as the reference value. For this purpose, several Drug Substances and Drug Products (Liquid formulation containing HSA, Liquid formulation HSA free) were analyzed. In all cases, the ratio Optimized / Current Interferon-beta-1a assay was not statistically different from 100%, which demonstrates that the two Interferon beta-1a assays generate statistically equivalent results.

In conclusion, the method has a better precision than the current assay, and generates accurate results statistically equivalent to those obtained with the current assay.

This invention has been described with reference to examples of both quantitative determination of proteins and purity assessment of the same in both SEC and RP-HPLC chromatographic methods.

It will be apparent that many other equivalent examples can be done without departing from the spirit and scope of the invention that is defined by the following claims.